Conservation Genet Resour (2018) 10:621–626
DOI 10.1007/s12686-017-0879-x

TECHNICAL NOTE

The complete mitochondrial genome and genetic distinction of the Taiwanese honeybee, *Apis cerana* (Hymenoptera: Apidae)

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Received: 1 September 2017 / Accepted: 24 September 2017 / Published online: 7 October 2017

Abstract The Asian cavity-nesting honeybee, *Apis cerana* is widely distributed across Asia and nearby islands, showing interesting patterns of genetic differences caused by repeated isolation and re-unification of populations owing to repeated changes in sea levels. In the present study, we analyzed the complete mitochondrial genome of *A. cerana* from Taiwan and eastern China for the first time. The mitochondrial genomes of these honeybee populations were circular 15,251- and 15,332-bp molecules, respectively, and included 13 protein-coding genes (PCGs), 22 tRNA genes, two rRNA genes, and one AT-rich control region. The average AT content in mitochondrial genome of Taiwanese and Chinese honeybees was 83.50 and 83.54%, respectively. The heavy strand encoded nine PCGs and 14 tRNA genes and the light strand encoded four PCGs, eight tRNA genes, and two rRNA genes. The *ATP6* and *ATP8* genes shared 19 nucleotides. Eight PCGs of the *A. cerana* mitochondrial genome started with ATT, *ATP6, COIII*, and *Cytb* genes with ATG, *ATP8* gene with ATC, and *ND4* gene with ATA. All tRNA genes formed typical cloverleaf secondary structures, except for *tRNA-Ser* (*AGN*). The phylogenetic analysis inferred from the 13 mitochondrial PCGs, based on maximum likelihood, indicated that the Taiwanese and eastern Chinese populations of *A. cerana* are closely related taxa. The 272 sites that differed between *A. cerana* from Taiwan and eastern China were evenly distributed throughout the mitochondrial genome. We found that the genetic distance between the two population was 0.025, indicating that they are genetically different enough to be considered different subspecies or local populations.

Keywords Asian honeybee · Genetic distance · Local population · Subspecies · Subcluster

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Taiwan Archipelago, located in East Asia, has an area of approximately 36,193 km^2, and 19% of its area is a nature reserve. Taiwan Archipelago was isolated from the Asian continent after the last glacial period. Many animals and plants inhabit its tropical to temperate biomes because annual rainfall exceeds 2500 mm and many mountains with elevation over 3000 m above sea level are present.

The Asian cavity-nesting honeybee, *Apis cerana* is widely distributed from central to eastern Asia, and the surrounding islands, including Taiwan. Multivariate morphometric analysis of *A. cerana* suggested the presence of four subspecies *A. cerana cerana, A. cerana indica, A. cerana himalaya*, and *A. cerana japonica* or six subclusters (morphoclusters) in Asia (Ruttner 1988; Radloff et al. 2010). The *A. cerana* of mainland China is morphologically divided into three clusters: (1) an "Aba" group in southern Ganshu, central and northern Sichuan provinces, and northern China; (2) a subcluster in central and eastern China; (3) a "southern" *A. cerana* subcluster in southern Yunnan, Guangdong, Guangxi, and Hainan in China (Tan et al. 2008; Radloff et al. 2010). Radlhoff et al. (2010)

showed results of at least three subclusters the morphological traits of *A. cerana* from mainland China, but *A. cerana* of Taiwan Archipelago unanalyzed. Mitochondrial DNA analyses of the partial DNA sequences indicated that *A. cerana* from China has higher genetic diversity than that in the populations from the Asian (Smith and Hagen 1996; Smith et al. 2000; Hepburn et al. 2001; Tan et al. 2007, 2016; Zhao et al. 2014, 2017). The non-coding region between *tRNA-Leu* and *COII* genes of mitochondrial DNA has revealed genetic variations among *A. cerana* populations a wide geographical range, spanning from India to Japan, and a unique haplotype (Taiwan short) found only in Taiwan (Smith and Hagen 1996; Smith et al. 2000; Takahashi et al. 2002, 2007). However, the phylogenetic relationship between the population from Taiwan and a subcluster in central and eastern China remains almost uncertain. Analysis based on the complete sequence of mitochondrial DNA of *A. cerana* provides more useful information on classification and phylogenetic relationship of local populations and subspecies (Takahashi et al. 2016; Okuyama et al. 2017a, b). In this study, we first analyzed the complete mitochondrial genome of *A. cerana* from the Taiwan and a subcluster in central and eastern of China populations to identify their phylogenetic position and genetic variation.

Adult workers of *A. cerana* in Taipei City, Taiwan and Suzhou City, Jiangsu, China were collected in May 2016 and April 2015, respectively. Genomic DNA was extracted from the thoracic muscle tissue using standard phenol/chloroform methods. We used two long PCR primer pairs (Okuyama et al. 2017a) and 27 cycle sequencing primers (Table 1) for the *A. cerana* mitogenome. The mitochondrial genomes of *A. cerana japonica* (AP017314) and *A. cerana* (GQ162109) were used as reference sequences (Tan et al. 2011a, b; Takahashi et al. 2016). The resultant reads were assembled and annotated using the MITOS web server (Germany; Bernt et al. 2013) and Geneious R9 (Biomatters, New Zealand). A phylogenetic tree was constructed using MEGA6 (Tamura et al. 2013) and TREEFINDER v.2011 (Jobb et al. 2004) based on the nucleotide sequences of the 13 protein-coding

Table 1 Cycle sequencing primer sequences in *Apis cerana*

Primer name	Direction	Sequence 5′ to 3′	Length	Genome location[a]
Location*AcmtDNA213intR	R	AATTTATCGACGGGGTATG	19	213
AcmtDNA820intF	F	TCTCCAAATAAGATCCCAAGA	21	820
AcmtDNA842intR	R	AATCTTGGGATCTTATTTGGAG	22	842
AcmtDNA1614intR	R	CGCAAATAAACTGTAAGCAAAG	22	1614
AcmtDNA2122intR	R	CCAAAACCTCCAATTA	16	2122
AcmtDNA2932intF	F	CAATTGGAGGATTAACAGG	19	2932
AcmtDNA4227intF	F	ACCAATCATAGTAGAATCTACATC	24	4227
AcmtDNA5067intR	R	GAATTGATAATGTTCATGGTCGA	23	5067
AcmtDNA5687intF	F	TTAGTTTCATCCGGTATTGC	20	5687
AcmtDNA5895intR	R	AGTCCATGAAATCCTGTTGC	20	5895
AcmtDNA6297intF	F	AAAAGATCACCATTTG	16	6297
AcmtDNA6461intR	R	TGAGAGTATTGTTCTATAAG	20	6461
AcmtDNA6892intF	F	CTTGGAATCTTTAATCTATG	20	6892
AcmtDNA7324intF	F	CGAAATGAATATGAAACTG	19	7324
AcmtDNA7436intF	F	CAACTAAAAATGGAAATCCACA	22	7436
AcmtDNA7455intR	R	TGGATTTCCATTTTTAGTTGG	21	7455
AcmtDNA8843intR	R	TTCATCAAATATAGGGTCACCA	22	8843
AcmtDNA9483intF	F	GATAATCAACGATTTTCTCTATACCC	26	9483
AcmtDNA9926intF	F	TTTTTGATGCCCTAATTCAT	20	9926
AcmtDNA11248intF	F	TTATCAAGTGTATGAGG	17	11,248
AcmtDNA11270intR	R	CCAATTCCTCATACACTTG	19	11,270
AcmtDNA11431intR	R	TTGAAAATCCACCTCAAATTC	21	11,431
AcmtDNA11775intF	F	CCCAAATAAACTTGGTGGAG	20	11,775
AcmtDNA12488intR	R	TGATTGAGGGAGAATCTGAA	20	12,488
AcmtDNA12729intF	F	CAACAAACAAAACTGGATAAAC	22	12,729
AcmtDNA14578intR	R	GTAAAAGTACTGGAAAGTG	19	14,578
AcmtDNA14931intF	F	TGGTTCAACATTTAATAGC	19	14,931

[a]The position of the 5′ end of 27 primers applied the position of Taiwanese *Apis cerana* (AP017984)

genes (PCGs) found in the complete mitochondrial genome sequences of honeybees used in this study and those present in GenBank.

The mitochondrial genome of *A. cerana* from Taiwan and Eastern China included 13 PCGs, 22 tRNA genes, 2 rRNA genes, and one AT-rich control region in a closed loop of 15,251 bp (AP017984) and 15,332 bp (AP017983) (Fig. 1). These results were similar to honeybee genomes (Tan et al. 2011a, b; Takahashi et al. 2016; Okuyama et al. 2017a, b). The AT content of the mitochondrial genome in the Taiwanese and Eastern Chinese populations of *A. cerana* was approximately 83.50 and 83.54%, respectively. Similar to the mitochondrial genomes of *A. cerana* group, the heavy strand encoded nine PCGs and 14 tRNA genes, and the light strand encoded four PCGs, eight tRNA genes, and two rRNA genes (Table 2). The initiation codon for the eight PCGs was ATT, which was ATG for *ATP6, COIII*, and *Cytb*, ATC for *ATP8*, and ATA for *ND4*. The stop codon for all PCGs was TAA. The *ATP8* and *ATP6* genes shared 19 nucleotides. All tRNA genes possessed cloverleaf secondary structures, except for *tRNA-Ser* (*AGN*), which lacked the dihydrouridine arm and occurred commonly in the Taiwanese and Eastern Chinese *A. cerana*. The *LrRNA* of Taiwanese *A. cerana* was 4 bp longer than that in Eastern China. The *ND6* gene and *SrRNA* of *A. cerana* was 6 and 2 bp longer in the Eastern Chinese than in the Taiwanese population (Table 1). The complete sequence of mitochondrial DNA in the Taiwanese population was shorter than that in the Chinese population, because the non-coding region between *tRNA-Leu* and *COII* was shorter in the former (Fig. 1).

The mutation sites and genetic distance of 13 PCGs of the mitochondrial genome between the Eastern Chinese and Taiwanese populations were 272 and 0.025, respectively. This result is consistent with the genetic distance generally observed among the complete mitochondrial genomes of *A. cerana* subspecies than within subspecies (Takahashi et al. 2016; Okuyama et al. 2017a, b). The phylogenetic analysis suggested that *A. cerana* from Taiwan was most closely related to *A. c. cerana* of eastern China among the *A. cerana* subspecies group (Fig. 2). The haplotype of the non-coding region between *tRNA-Leu* and *COII* genes was a unique short sequence, which was consistent with the findings of previous studies (Smith and Hagen 1996; Smith et al. 2000; Takahashi et al. 2002, 2007). We confirmed that *A. cerana* of Taiwan is genetically isolated from those of China or the other populations, and that it has genetic differences at the subspecies rank as compared with the *A. cerana cerana*. These results suggest that the Taiwanese population is an independent subspecies. We

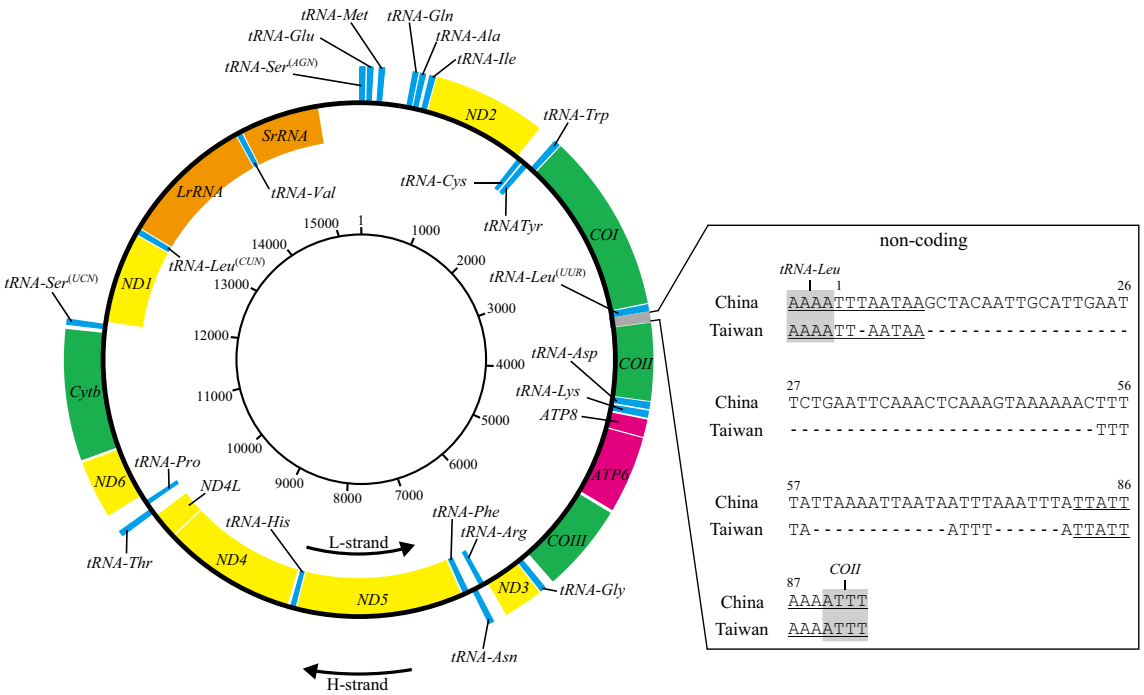

Fig. 1 Physical map and sequences of the non-coding intergenic region between *tRNA-Leu* and *COII* of the mitochondrial genome of the Taiwanese honeybee *Apis cerana*. Genes illustrated on the outside of the main circle are encoded on the heavy (H) strand; genes on the inside of the circle are encoded on the light (L) strand. The *ND1-ND6* genes are labeled in yellow, *COI, COII, COIII* and *Cytb* genes are labeled in green, *ATP8* and *ATP6* genes are labeled in pink, 22 tRNA genes are labeled in blue, and *LrRNA* and *SrRNA* genes are labeled in orange. Gaps are shown by dashes. The underlined indicate the "stem" of the hairpin structure. (Color figure online)

Table 2 Mitochondrial genome organisation of *Apis cerana* from Taiwan and China

Gene/region	Strand[a]	Anticodon	Codon		Taiwan (AP017984)		China (AP017983)		Mutaion sites (indel)	Genetic distance
			Start	Stop	Start–stop	Size (bp)	Start–stop	Size (bp)		
tRNA-Ser (*S1*)	H	TCT	–	–	1–60	60	1–60	60		
tRNA-Glu (*E*)	H	TTC	–	–	64–129	66	64–129	66		
tRNA-Met (*M*)	H	CAT	–	–	164–229	66	164–229	66		
tRNA-Gln (*Q*)	H	TTG	–	–	470–531	62	459–520	62		
tRNA-Ala (*A*)	H	TGC	–	–	532–597	66	521–586	66		
tRNA-Ile (*I*)	H	GAT	–	–	617–682	66	605–670	66		
ND2	H	–	ATT	TAA	683–1678	996	671–1666	996	20	0.0201
tRNA-Cys (*C*)	L	GCA	–	–	1678–1743	66	1666–1731	66		
tRNA-Tyr (*Y*)	L	GTA	–	–	1749–1817	69	1737–1805	69		
tRNA-Trp (*W*)	H	TCA	–	–	1834–1902	69	1822–1890	69		
COI	H	–	ATT	TAA	1903–3468	1566	1891–3456	1566	37	0.0236
tRNA-Leu (*L2*)	H	TAA	–	–	3464–3533	70	3452–3521	70		
COII	H	–	ATT	TAA	3559–4239	681	3611–4291	681	15	0.0220
tRNA-Asp (*D*)	H	GTC	–	–	4238–4305	68	4290–4357	68		
tRNA-Lys (*K*)	H	TTT	–	–	4312–4383	72	4364–4435	72		
ATPase8	H	–	ATC	TAA	4390–4551	162	4442–4603	162	4	0.0247
ATPase6	H	–	ATG	TAA	4533–5210	678	4585–5262	678	24	0.0354
COIII	H	–	ATG	TAA	5228–6007	780	5280–6059	780	29	0.0372
tRNA-Gly (*G*)	H	TCC	–	–	6048–6114	67	6126–6192	67		
ND3	H	–	ATT	TAA	6115–6468	354	6193–6546	354	10	0.0282
tRNA-Arg (*R*)	L	TCG	–	–	6488–6552	65	6566–6631	66		
tRNA-Asn (*N*)	H	GTT	–	–	6572–6639	68	6651–6718	68		
tRNA-Phe (*F*)	L	GAA	–	–	6658–6728	71	6737–6807	71		
ND5	L	–	ATT	TAA	6735–8402	1668	6814–8481	1668	38	0.0228
tRNA-His (*H*)	L	GTG	–	–	8403–8468	66	8482–8547	66		
ND4	L	–	ATA	TAA	8487–9815	1329	8565–9893	1329	32	0.0241
ND4L	L	–	ATT	TAA	9816–10,079	264	9894–10,157	264	5	0.0189
tRNA-Thr (*T*)	H	TGT	–	–	10,103–10,169	67	10,181–10,247	67		
tRNA-Pro (*P*)	L	TGG	–	–	10,185–10,263	79	10,263–10,340	78		
ND6	H	–	ATT	TAA	10,315–10,821	507	10,391–10,903	513	15(6)	0.0296
Cyt b	H	–	ATG	TAA	10,833–11,981	1149	10,916–12,064	1149	32	0.0279
tRNA-Ser (*S2*)	H	TGA	–	–	12,005–12,071	67	12,088–12,154	67		
ND1	L	–	ATT	TAA	12,084–12,998	915	12,167–13,081	915	13	0.0142
tRNA-Leu (*L1*)	L	TAG	–	–	12,999–13,067	69	13,082–13,150	69		
LrRNA	L	–	–	–	13,068–14,399	1332	13,151–14,478	1328	20(4)	0.0151
tRNA-Val (*V*)	L	TAC	–	–	14,400–14,466	67	14,479–14,545	67		
SrRNA	L	–	–	–	14,467–15,251	785	14,546–15,332	787	9(2)	0.0115

[a]*H* heavy strand, *L* light strand

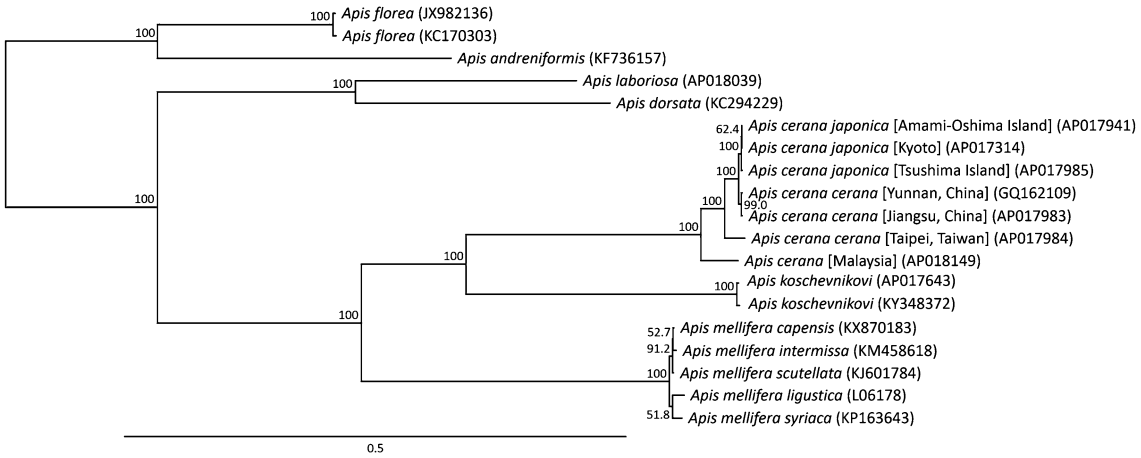

Fig. 2 Phylogenetic relationships (maximum likelihood) of species of the genus *Apis* (Hymenoptera) based on the nucleotide sequence of the 13 protein-coding genes in the mitochondrial genome. These sequences were separated by the codon positions, and for each partition, optimal models of sequence evolution were used in maximum likelihood method in TREEFINDER based on the corrected Akaike information criterion (AICc). Numbers beside each node represent bootstrap values in percentage based on 1000 replications. *Apis florea* was used as an outgroup. Alphanumeric terms in parentheses indicate GenBank Accession Numbers

conclude that it is necessary to investigate Taiwan and neighboring populations morphologically and genetically.

Acknowledgements We are grateful to Prof. Yoshitaka Tsubaki, Mr. Takeshi Wakamiya and Miss. Yunnna Yoshioka for kindly providing data for helping our analysis.

Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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